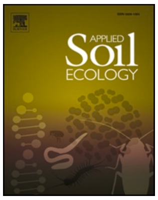
Applied Soil Ecology 206 (2025) 105881

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil



Research paper

Disturbance from tillage is a dominant factor in explaining differences in soil biodiversity of three grasslands management types

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ARTICLE INFO

Keywords:
Permanent grasslands
Multi-species grasslands
Semi-natural grasslands
Soil biota
Microbial
Nematodes
micro-arthropods
Earthworms
Regenerative agriculture

ABSTRACT

Intensification of agriculture has reduced both aboveground and belowground biodiversity, as well as their ecosystem services. A transition towards regenerative agricultural systems is supposed to enhance functional agro-biodiversity and create more resilient systems. For dairy or beef farming, extensification towards semi-natural grasslands and establishing multi-species grasslands are potential contributors to this transition. Both grassland types differ in terms of plant diversity and management, but it is unclear whether and how these differences translate into soil quality and soil biodiversity. An on-farm trial was set up in which the abundance and diversity of soil biota was compared between intensively managed species-poor permanent grasslands, intensively managed frequently re-seeded multi-species grasslands, and extensively managed species-rich semi-natural grasslands. Total abundance and biomass of nematodes, micro-arthropods and earthworms did not differ between these grassland types. Bacterial PLFA was higher in semi-natural grasslands than in multi-species grasslands; intensive-permanent grasslands took an intermediate position. Protozoan PLFA was higher in intensive-permanent grasslands than in multi-species grasslands; semi-natural grasslands took an intermediate position. The grassland management types only had an effect on alpha and gamma diversity in the case of micro-arthropods, with a higher taxonomic richness in semi-natural grasslands. Nematode, micro-arthropod and earthworm taxonomic community structures differed between grassland management types. Differences in abundance and diversity of soil biota between multi-species grasslands on the one hand and intensive-permanent and semi-natural grasslands on the other hand were thought to be mostly related to disturbances caused by tillage and their direct and indirect effects on soil biota and its living conditions. Consequently, longevity without regular soil disturbance is considered important for the transition to grassland systems such as multi-species grasslands that simultaneously utilise and support soil biodiversity and its ecosystem services. However, what must also be considered in decisions on the transition to future farming systems are the impacts on aboveground biodiversity (including insects and birds) and ecosystem services such as agricultural production.

1. Introduction

Agriculture faces the triple challenge of an increasing global demand for food while maintaining ecosystem services against the backdrop of the urgency to increase the resilience of production systems in an ever more volatile environment. At present this is off-kilter: the increasing demand for food has led to intensification which reduces aboveground (Rigal et al., 2023) and belowground biodiversity (Tsiafouli et al., 2015), and impairs their ecosystem services, thus making agriculture more vulnerable to stress such as climate change (De Vries et al., 2012). For example, whereas complex fungal-based food webs of extensive systems are quite resistant to drought (De Vries et al., 2012), the loss of belowground biodiversity could result in simplified soil food webs with impaired functions (Chomel et al., 2022). A transition is needed to agricultural practices that simultaneously use and support biodiversity and ecosystem services. Regenerative or nature-inclusive agricultural

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https://doi.org/10.1016/j.apsoil.2025.105881
Received 20 April 2024; Received in revised form 6 January 2025; Accepted 9 January 2025

systems are supposed to enhance functional biodiversity and create more resilient systems in which both food production and nature can thrive (Erisman et al., 2015, 2016; Schreefel et al., 2020).

For dairy or beef farming, potential components of making agricultural systems more nature-inclusive could either be an extensification of intensively-used permanent grasslands towards semi-natural grasslands, or establishing multi-species grasslands in which specific functional plant species are sown (Van Eekeren and Visser, 2019; Isbell et al., 2011). In the EU, permanent grassland is defined as land used to grow grasses, which is not included in the crop rotation for five years or longer (Commission Regulation EU No 796/2004). Semi-natural grasslands are defined as managed ecosystems dominated by native or naturally-occurring grasses and other herbaceous species (Allen et al., 2011). In the Netherlands these grasslands are mostly restored from the natural occurring seedbank with an extensive management of grazing or mowing (maximum twice a year) and maximum fertilisation of 10 ton of farm yard manure per ha (Van Eekeren and Visser, 2019). Multi-species grasslands support functional biodiversity as they include a selection of grasses, legumes and forbs which are sown by the farmer (not necessarily native local species). In the Netherlands, multi-species grasslands contain a minimum of eight legumes and forbs, are fertilised with mainly slurry manure and are intensively used for grazing and mowing (Van Eekeren and Visser, 2019). Particularly the presence of legumes facilitates nitrogen availability for the vegetation and increases dry matter yield, compared to grasslands without legumes (Sanderson et al., 2005; Høgh-Jensen et al., 2006; Llurba et al., 2010; Hoekstra et al., 2018; De Haas et al., 2019).

The transition from species-poor grasslands to semi-natural grasslands or multi-species grasslands will result in a change in plant diversity and grassland management; however, it remains unclear how this combination of plant diversity and management will translate into soil quality and soil biodiversity. This transition will contribute to an increased diversity in plant roots and root traits such as root length and exudation, which improves soil structure and soil organic carbon cycling (Lange et al., 2015; Gould et al., 2016; El Moujahid et al., 2017). This may also influence the resource quantity, quality and stability for soil biota. For specific soil biota, it is not only the diversity of plant species but also the identity of the plant species which is important (De Deyn et al., 2011; Eisenhauer et al., 2012; Lange et al., 2015; Gould et al., 2016). For example, the presence of legumes was found to increase earthworm abundance and soil faunal activity (Gastine et al., 2003; Van Eekeren et al., 2009a; Crotty et al., 2015), which in turn was linked to increased soil water dynamics and soil structure (Van Eekeren et al., 2009a, 2010; Deru et al., 2018).

Besides management (e.g. mowing regime and fertilisation level) aiming to increase plant species diversity, multi-species grasslands often undergo specific soil management that can affect soil biota and belowground processes. To maintain species composition in multi-species grasslands, grassland renewal occurs more frequently (once every four to five years) than in permanent grasslands (on average once every ten years in intensively-managed permanent grasslands (Velthof et al., 2009)) and semi-natural grasslands (no grassland renewal). Such grassland renewal is often accompanied by some form of tillage, with negative effects on the abundance, biomass and diversity of soil biota and consequently reduced microbial activity (CO_2 respiration) (Van Eekeren et al., 2008). In contrast, permanent and semi-natural grasslands are less frequently renewed or not at all, and the latter are not fertilised either. In permanent and multi-species grasslands, nutrient input comes from fertilisation with organic or inorganic fertiliser, or from N-fixation by leguminous plants. It has been found that inorganic fertiliser use significantly alters soil biodiversity as it causes lower soil faunal and arbuscular mycorrhizal fungal diversity and increased bacterial diversity (Lin et al., 2013; De Graaff et al., 2019). Broadly speaking, a distinction can be made between nutrient-enriched grassland systems that support high levels of herbivory, and nutrient-poor grassland systems that support low levels of herbivory. Ecosystems dominated by plant species that are adapted to nutrient rich conditions have high litter quantity and quality, and they support soil food webs with a bacteria-based energy channel and dominant microfauna (i.e. nematode and protozoa) and earthworm populations. In contrast, nutrient-poor soil conditions support plants with low litter quantity and quality, and they tend to have soil food webs dominated by fungi and mesofauna (e.g. collembola and acari) (Swift et al., 1979; Wardle, 2002; Wardle et al., 2004).

The aim of our study was to investigate on-farm the difference in the abundance and diversity of several soil biota simultaneously between intensively-managed species-poor permanent grasslands, intensively-managed frequently re-seeded multi-species grasslands, and extensively managed species-rich semi-natural grasslands. We selected twelve blocks with each block having a representation of the three grassland management types (a) intensively-managed permanent grassland, (b) recently-sown multi-species grassland, and (c) semi-natural grassland (in total 36 field). We hypothesised that (i) the abundance of bacteria, nematodes and earthworms is highest in the intensively-management permanent grasslands and in the recently-sown multi-species grasslands, and that the fungal biomass and the abundance of microarthropods is highest in the semi-natural grasslands, (ii) the alpha diversity is higher in grasslands with a higher number of years since last tillage, whereby the alpha diversity is higher in semi-natural grasslands than in intensively-managed permanent grasslands, (iii) the gamma diversity is highest in semi-natural grasslands, and (iv) consequently the soil food web in intensively-managed permanent and multi-species grasslands supports more herbivory and a bacterial-based food web with a predominance of microfauna and earthworms, while the soil food web of the semi-natural grasslands is dominated by a fungal-based food web and mesofauna.

2. Materials and methods

2.1. Experimental design and sites

On the sandy soils in the East of the Netherlands (Achterhoek, Province of Gelderland) (see Supplementary Fig. S1), twelve replicates were selected for each of the following three grassland management types:

- Intensively-managed permanent grasslands with at least five years since last tillage and sowing (following the definition of permanent grassland of the EU (Commission Regulation EU No 796/2004)), at least 80 % vegetation cover of perennial rye grass (*Lolium perenne L.*) and no >5 % of legumes, and conventional management with a fertilisation of slurry manure and inorganic fertiliser.
- Intensively-managed multi-species grasslands that were sown at least one year ago with a mixture of grasses and a minimum of eight different species of legumes and forbs, and conventional management with a fertilisation of mainly slurry manure. Grasslands were selected which were successfully established with most of the species sown present.
- Extensively-managed semi-natural grasslands that have been without tillage for >10 years, no fertilisation, no herbicides, and a plant diversity of at least 15 plant species per 25 m^2.

All grasslands were selected in blocks in which the three grassland management types were represented (randomised complete blocks). Within a block the grasslands were located between 250 m and 6 km from each other to limit climatic variability as much as possible. All grasslands within the same block were selected to have the same sandy soil type (Typic Haploquod or Plaggeptic Haploquod; Soil Survey Staff, 1999) and landscape structures (i.e., open landscape or hedgerows). Table 1 presents an overview of the management on the selected grasslands of the three grassland management types in the year of soil sampling (2021).

Table 1

Management parameters for intensive-permanent, multi-species and semi-natural grasslands ($n = 12$ for each management type). ANOVA p-values are given, with significant results ($p < 0.05$) in bold. Parameter means with different letters indicate significant differences according to post-hoc Tukey tests ($p < 0.05$).

		Grassland management type						
		Intensive-permanent		Multi-species		Semi-natural		
Parameter	Unit	Mean	s.d.	Mean	s.d.	Mean	s.d.	p-value
Field size	Ha	3.52	1.91	2.43	0.63	1.83	1.27	0.067
Number of years since last tillage	Years	8.3^b	5.3	1.4^a	0.5	21.3^b	14.3	**<0.001**
Number of grass cuts*	n yr^{-1}	7^b	1	6^b	1	2^a	3	**<0.001**
Mineral N applied from manure	kg N ha^{-1} yr^{-1}	123^c	27	66^b	34	1^a	2	**<0.001**
N applied from inorganic fertiliser	kg N ha^{-1} yr^{-1}	155^c	41	29^b	32	0^a	0	**<0.001**
Lolium perenne	% cover	79.7^c	7.9	61.1^b	20.7	19.3^a	25.8	<0.001
Leguminous species	% cover	5.5^a	3.9	21.8^c	3.4	9.6^b	4.1	**<0.001**
Plant species richness	N	16.9^a	3.1	17.0^a	3.6	29.3^b	4.5	**<0.001**
Plant ENS** (N1)	N	8.2^a	2.4	11.3^b	2.5	18.5^c	3.8	**<0.001**

[*] Cuts – Cumulative mowing and grazing cuts. For semi-natural grasslands this was mainly mowing, aftermath grazing was practiced after mowing on only 3 of the 12 grasslands.

[**] ENS, effective number of species based on Hills number N1, i.e. the exponent of the Shannon Index.

The Tansley vegetation survey method (Tansley et al., 1927; Tansley, 1946) was used to determine plant species cover on an area of 5000 m^2 in the centre of the grasslands. The timing of the survey followed the development of the vegetation of the different grassland management types and took place from April to July 2021. Categories of cover were altered to species abundance for statistical analysis according to Hennekens (2020). The plant species composition is part of the experimental design, and the findings are shown in Table 1. Semi-natural grassland plant species richness was significantly higher ($p < 0.001$) compared to both other management types, and it was categorised in the Eunis habitat types as E2 Mesic grasslands + E3 Seasonally wet and wet grasslands. The cover of *Lolium perenne* was highest in permanent grasslands and lowest in semi-natural grasslands while the cover of leguminous species was highest in the multi-species grasslands and lowest in the intensive-permanent grasslands. Plant diversity (Effective Number of Species, ENS) was more than twice as high in semi-natural grasslands than in intensive-permanent grasslands, with the multi-species grasslands in between these two. Of the 18 plant species that the different grasslands had in common, 9 species were only found in intensive-permanent and semi-natural grasslands, and 9 species were only detected in the multi-species grasslands and semi-natural grasslands. Four plant species were exclusive to intensive-permanent grasslands, 12 to multi-species grasslands, and 47 to semi-natural grasslands.

Weather data were taken from weather stations from the Royal Netherlands Meteorological Institute near the region of the experiment (KNMI - Dagwaarden Neerslagstations, 2022; KNMI - Klimaatviewer, 2021a; KNMI - Klimaatviewer, 2021b). In the growing seasons of 2021, average air temperature was 12.9 °C, while the 30-year average (1991–2020) was 10.1 °C. Total rainfall during the growing season 2021 was 574 mm, while the 30-year average was 540 mm. The 2021 growing season was considered a normal year.

2.2. Soil sampling

Our objective was to measure longer-term effects of the grassland management types rather than growth-seasonal related management effects. Consequently, all abiotic and biotic soil samples were taken at the end of the growing season between 15 and 18 November 2021 rather than at the start of the growing season, so as to avoid direct effects of fertilisation on soil biota. All abiotic and biotic soil samples were taken from a 5 × 5 m surface representative of the field and within the 5000 m^2 area which was surveyed for vegetation. From the 5 × 5 m area, a composite sample consisting of 50 cores (0–10 cm depth, ⌀ 2.3 cm) was collected, sieved through 1 cm mesh, homogenised, and stored at 4 °C until analysis. The composite sample was split into sub-samples for abiotic and biotic (microbiological and nematode) analysis (see 2.3 and 2.4). For soil organic matter (SOM) and total carbon, samples were taken from 0 to 30 cm depth, sieved through 1 cm mesh, homogenised, and stored at 4 °C until analysis. Separate samples were collected for microarthropods and earthworms (for details, see 2.4.3 and 2.4.4, respectively).

2.3. Abiotic soil parameters

The pH of the oven-dried soil samples was measured in 1 M KCl (pH-KCl). Soil organic matter (SOM) was determined by loss-on-ignition at 550 °C for 3 h (Ball, 1964). Total Carbon (C) was measured by incineration of dry material at 1150 °C, after which the CO_2 produced was determined by an infra-red detector (LECO Corporation, St. Joseph, Mich., USA). The phosphorous fraction with ammonium-lactate (P_{Al}) was determined according to the standard method described in Bronswijk et al. (2003). Total potassium (K) in solution was determined using flame photometry after extraction of soil with HCl (0.1 M) and oxalic acid (0.5 M) in a 1:10 m:v ratio and filtration (Bronswijk et al., 2003).

Field water content and bulk density were measured in the 5–10 cm layer below the soil surface, in three undisturbed ring samples containing 100 cm^{-3} soil. Samples were weighed, oven-dried (70 °C), and re-weighed to determine water content and bulk density (McKenzie et al., 2004).

2.4. Biotic soil parameters

2.4.1. Microbes

Bacterial, fungal, actinomycete and protozoan biomass was determined by Eurofins (Wageningen, the Netherlands), using phospholipid fatty acids (PLFA) analysis. PLFA are the primary lipids composing the membranes of living cells. For the extraction, 4 g of a field-moist soil sub-sample from the composite soil sample was used (see 2.2). The PLFA were separated on a GC Trace 1300 and analysed on a TSQ 8000 mass spectrometer (20 m × 0.15 mm ID, 0.30 μm VF-5MS Agilent (Agilent, Santa Clare, United States); 1 μl injection; helium; full scan 50–300 m/z) using a temperature gradient. The retention time of the different phospholipids and the mass spectrum was used for determination of the lipids. A compound database and internal standards of Eurofins were used to determine any shifts in retention time. The analysis was performed according to standard methods NPR-CEN-ISO/TS 29843–1 and NPR-CEN ISO/TS 29843–2. PLFA 10Me-16:0, 10Me-17:0, 10Me-18:0, 12Me-18:0, i15:0, ai15:0, i16:0, ai16:0, 16:1ω7c, 17:1w8c, i17:0, i17:0 ω7c, ai17:0, cy17:0, 18:1ω7c, 18:1ω9t, 18:1ω12t, cy19:0ω7c and cy19:0ω9c were chosen to represent bacteria, and PLFA 18:2ω6 was used as a marker of saprotrophic fungi. PLFA 10Me16:0, 10Me17:0 and 10Me18:0 were used as markers of actinomycetes, and PLFA 20:4ω6c was used for protozoa.

Potentially mineralisable N was determined by anaerobic incubation of a soil sample under water for 1 week at 40 °C (Keeney and Nelson, 1982; Canali and Benedetti, 2006).

2.4.2. Nematodes

Nematodes were extracted from a 100 g field-moist sub-sample of the composite soil sample (see 2.3), using an Oostenbrink elutriator and subsequent Bearman extraction on cotton wool filters (Oostenbrink, 1960). Total numbers were counted and expressed per 100 g fresh soil. Nematodes were fixed in hot formaldehyde (4 %), and at least 150 randomly selected nematodes from each sample were identified to genus and assigned to trophic groups. Additionally, the carbon footprint of trophic groups, and the nematode channel, enrichment and structure index (Ferris et al., 2001) were calculated using the online NINJA tool (https://shiny.wur.nl/ninja/; Sieriebriennikov et al., 2014; Ferris, 2010).

2.4.3. Micro-arthropods

Per field, three samples for micro-arthropods were collected with a core sampler of 7.5 cm length and a diameter of 5.8 cm, holding 3 PVC rings of 2.5 cm high. Micro-arthropods were extracted by placing the soil cores in a Tullgren funnel. The temperature in the upper compartment of the funnel was set at 30 °C and in the lower part the temperature was kept at 5 °C. The organisms moved downwards to escape the heat, moved through a mesh, and were collected in a bottle containing 70 % ethanol. The total extraction time was one week (Siepel and Van de Bund, 1988). A sub-sample was taken via the gel-based sub-sampling method described by Jagers op Akkerhuis et al. (2008). Collembola and mites were counted separately and 70–100 randomly selected collembola and mites were identified to family and were possible to genus and species, and assigned to feeding guilds (Siepel, 1995).

2.4.4. Earthworms

Earthworms were sampled in two soil cubes (20 × 20 × 20 cm) per field in the 0–20 cm soil layer. After transporting the soil cubes to the laboratory, earthworms were hand-sorted, counted, weighed (without emptying their guts), and fixed in alcohol prior to identification. Abundance and biomass were expressed per m^2. All specimens, i.e. adults and juveniles, were identified to species (Sims and Gerard, 1985; Stöp-Bowitz, 1969), and classified into functional groups (epigeic, endogeic and anecic species) (Bouché, 1977). Our method, using soil cubes of up to 20 cm depth, leads to underrepresentation in the sample of anecic species such as *Lumbricus terrestris*.

2.5. Statistical analyses

All statistical analyses were performed in R version 4.1.1 (R Core Development Team, 2021). To test the differences between the three grassland management types for all measured parameters, one-way blocked ANOVA tests were carried out. For each parameter, data residuals were first tested for normality and homoscedasticity by fitting a linear model and visually checking the model plots. Parameters with binomial and equally varied residuals were then fitted in linear mixed effect (LME) models with block as a random factor using the *lme* function from the *nlme* package version 3.1–152 (Pinheiro et al., 2021). When heteroscedasticity of a parameter was suspected, Bartlett's test was carried out, using the *bartlett.test* function. If the outcome was significant, parameters were fitted with a generalised least squares (GLS) model which included a varIdent variance structure, in line with Zuur et al. (2009). This model structure, which is also included in the *nlme* package, allows for a different variation of residuals between treatments; therefore, it can be used with heteroscedastic data. Post-hoc Tukey HSD tests were used on significant ANOVA outcomes of all models to determine significant differences between treatments using the *glht* function of the *multcomp* package version 1.4–17 in R (Hothorn et al., 2021). Plant and earthworm species counts, as well as micro-arthropod family and nematode genus counts were used to calculate the Shannon species diversity index (H) (Shannon, 1948), using the diversity function in the package *vegan 2.5–7* (Oksanen et al., 2020). The exponent of Shannon's H was then calculated to give the effective number of species (ENS), which displays the number of equally-common species in a field and gives a more intuitive measure of diversity, as argued by Jost (2006). Nematodes that were identified to family were excluded from the diversity calculations to avoid overestimation of biodiversity. Alpha diversity refers to diversity on a local scale, describing in this research the average species diversity (richness) over one grassland management type. Gamma diversity describes the overall species diversity across communities within a larger geographical area, and in this research it describes the cumulative species diversity (richness) of all locations within a grassland management type (Andermann et al., 2022).

Pearson correlations were calculated for all pairs of parameters. The *vegan* package was used to carry out Principal Component Analysis (PCA) and Redundancy Analysis (RDA). PCA was carried out to visualise the variation explained by soil parameters and their correlations across sites. Variation between blocks was removed from the PCA by having block as a constraining variable without including it in the visualisation. RDA was conducted as a constrained ordination to determine the variation in the data that could be explained by the different forms of grassland management. RDA models were tested for significance through Monte Carlo permutation (999 permutations). Variation between blocks was conditioned for in the RDA model and permutation testing. As our focus was on the soil parameters, scaling 2 was used to create the PCA and RDA biplots (Borcard et al., 2011), and vegetation characteristics were not included in the analysis.

3. Results

3.1. Abiotic soil parameters

The pH_{KCl} was significantly lower in the semi-natural grasslands than in the other two grassland management types (Table 2). PAl did not differ, but plant-available K was significantly lower for semi-natural grasslands compared to the other two grassland management types. SOM and C_{total} in the 0–10 cm and 0–30 cm soil layer did not differ significantly over the three grassland management types. Both soil N_{total} and potentially-mineralisable N was highest for intensive-permanent grasslands and lowest for the multi-species grasslands ($p = 0.037$ and $p = 0.009$ respectively); semi-natural grasslands took an intermediate position. N_{total} was significantly correlated with SOM 0–10 cm (Supplementary Table S1). At the time of sampling, the moisture content in the multi-species grasslands was lowest. Soil bulk density was highest in the multi-species grasslands and lowest in the semi-natural grasslands.

3.2. Biotic soil parameters

3.2.1. Microbes

For total bacteria and actinomycetes, semi-natural grasslands had significantly higher amounts of PLFA than multi-species grasslands (Table 3), while intensive-permanent grasslands took an intermediate position for both groups. Both total bacterial and actinomycete PLFA were correlated with potentially mineralisable N ($r = +0.74$; $r = +0.69$, respectively), N-total ($r = +0.58$; $r = +0.56$) and number of years since last tillage ($r = +0.45$; $r = +0.34$) (Supplementary Table S1). Saprotrophic fungal PLFA tended to be higher in semi-natural grasslands than in multi-species grasslands ($p = 0.084$). The fungal-to-bacterial PLFA ratio was not significantly different between the three grassland management types. Intensive-permanent grasslands had the highest and multi-species grasslands the lowest amount of protozoan PLFA. Protozoan PLFA was correlated to saprothrophic fungal PLFA ($r = +0.62$, total bacterial PLFA ($r = +0.33$) and actinomycete PLFA ($r = +0.35$).

3.2.2. *Nematodes*

The nematode abundance did not differ significantly between the management systems (Table 4). Compared to the intensive-permanent and multi-species grasslands, the semi-natural grasslands had a significantly higher proportion of epidermal and root-hair feeders ($p = 0.009$), and a trend for a lower proportion of plant-feeding ectoparasites ($p = 0.070$). This translated into a lower herbivore carbon footprint in the semi-natural grasslands compared to the other two grassland types. There was a positive correlation between the carbon footprint of herbivores and aboveground biomass production (r = +0.51) measured in 2021 and 2022 by Zetterlind et al. (submitted) next to the area sampled for soil parameters. Both the intensive-permanent and semi-natural grasslands had a higher carbon fungivore footprint than the multi-species grasslands. The fungivore footprint was correlated with the amount of PLFA for saprotrophic fungi (r = +0.63) (Supplementary Table S1). Although no difference in nematode alpha and gamma diversity was found, the RDA of the nematode community showed a difference in community composition between the three grassland types (Fig. 1). The significant difference on the first RDA axis was mainly determined by herbivore taxa such as *Paratylenchus*, *Meloidogyne* and *Pratylenchus* in multi-species grasslands and predator taxa such as *Tripyla* in semi-natural grasslands. The first axis of the RDA of nematode genera was correlated with aboveground biomass production (r = +0.84) (Zetterlind et al., submitted), number of plant species ($r = -0.65$), number of years since last tillage ($r = -0.60$) and saprotrophic fungal PLFA ($r = -0.36$).

3.2.3. *Micro-arthropods*

The abundance of micro-arthropods did not differ between the three grassland management types (Table 5). The micro-arthropod alpha diversity and ENS were significantly higher in the semi-natural grasslands than in the other two grassland management types, and gamma diversity showed a similar trend. (Supplementary Table S2). The semi-natural grasslands also had a higher abundance of fungivorous grazers and herbivorous browsers. There was a significant correlation between the abundance of fungivorous grazers and the number of years since last tillage ($r = 0.43$) (Supplementary Table S1). The RDA of the micro-arthropod family abundance residuals showed a significant difference on the first RDA axis between semi-natural grasslands on the one hand and intensive-permanent and multi-species grasslands on the other (Fig. 2). This difference was mainly determined by the Phenopelopidae and Ceratozetidae (both fungivorous grazers) and the Pygmephoridae (fungivorous browsers), which were mainly present in semi-natural grasslands. Fungivorous grazers are able to digest both cell walls and cell contents while browsers are able to digest only cell contents (Siepel and De Ruiter-Dijkman, 1993). The first axis of the RDA of micro-arthropod families was correlated with aboveground biomass production (r = +0.75) (Zetterlind et al., submitted), number of years since last tillage (r = −0.6) and number of plant species ($r = -0.53$), with the semi-natural grasslands located on the negative side of the RDA axis ($p > 0.001$).

3.2.4. *Earthworms*

The total earthworm abundance and biomass did not differ between the three grassland management types (Table 6). Semi-natural grasslands had a significant higher abundance of epigeic earthworms than multi-species grasslands ($p = 0.031$); intensive-permanent grasslands took an intermediate position. There was no difference in the abundance of endogeic and anecic earthworms, but five of the intensive-permanent grasslands and four of the multi-species grasslands contained anecic

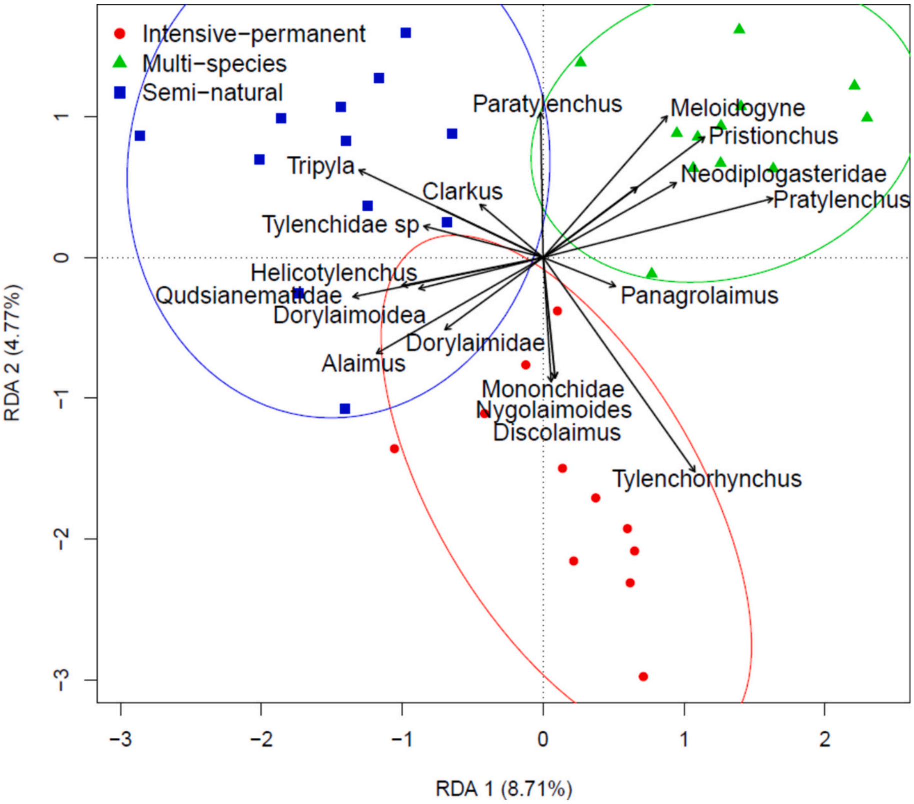

Fig. 1. RDA biplot displaying the first and second redundancy axes of the nematode genus and family abundance residuals (n 100 g^{-1} soil). Only species vectors that explained >5 % variation were included. Variation between blocks (31.33 %) has been conditioned for in the analysis. Grassland management types (intensive-permanent, multi-species, and semi-natural grasslands) are indicated by different colours and ellipses with 95 % confidence levels. Monte Carlo permutation testing showed that the model was significant ($p < 0.001$), and explained 61.00 % of total variation after conditioning. The first RDA axis was significant ($p < 0.001$) whereas the second axis was not considered significant in explaining total variation ($p = 0.122$).

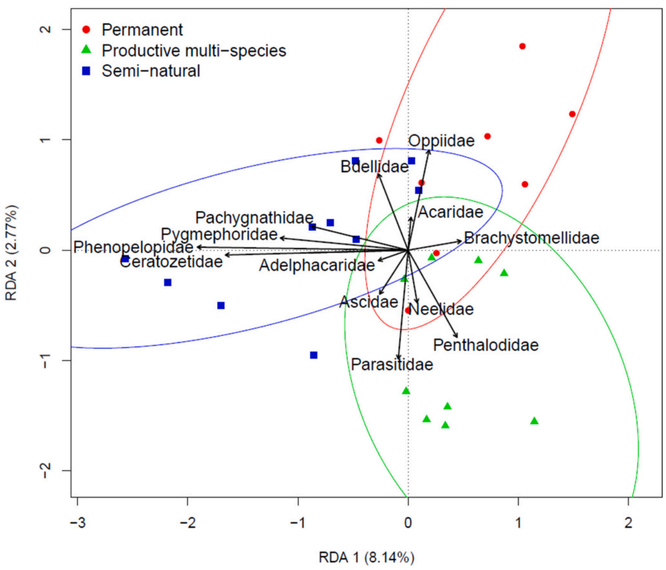

Fig. 2. RDA biplot showing the first and second redundancy axes of the micro-arthropod family abundance residuals. Only species vectors that explained >5 % variation were included. Variation between blocks (34.84 %) has been conditioned for in the analysis. Grassland management types (intensive-permanent, multi-species, and semi-natural grasslands) are indicated by different colours and ellipses with 95 % confidence levels. Monte Carlo permutation testing showed that the model was significant ($p = 0.017$), and explained 55.83 % of total variation after conditioning. The first RDA axis was significant ($p = 0.007$) whereas the second axis was not considered significant in explaining total variation ($p = 0.971$).

earthworms, while anecic earthworms were detected in only one of the semi-natural grasslands. In the RDA, a significant part of the variation in earthworm species composition could be related to the grassland management types (Fig. 3). The first RDA axis showed a significant difference between on the one hand semi-natural grasslands and on the other intensive-permanent and multi-species grasslands. This was mainly caused by two endogeic species; *Aporrectodea limicola* was more abundant in semi-natural grasslands whereas *A. calliginosa* and *A. nocturna* had a higher abundance in permanent and multi-species grasslands.

3.2.5. Alpha and gamma diversity of soil biota

The cumulative alpha diversity for earthworms, nematodes and micro-arthropods was highest in semi-natural grassland (41.4 genera), followed by multi-species grasslands (36.3 genera) and intensive-permanent grasslands (35.9 genera) (Tables 4, 5 and 6). Gamma diversity of earthworms, nematodes and micro-arthropods was highest in semi-natural grasslands (94 taxa), followed by intensive-permanent grasslands (83 taxa), and multi-species grasslands (80 taxa) (Fig. 4). Differences were mainly caused by micro-arthropods, the only taxon with a statistically different taxon richness between the grassland management systems (Table 5). There were 17 unique taxa for semi-natural grasslands, 6 unique taxa for multi-species grasslands and 4 unique taxa for intensive-permanent grasslands. Differences in unique taxa were mainly determined by differences in micro-arthropod families.

4. Discussion

4.1. Microbes

We hypothesised a positive relationship between fertilisation rates and bacterial PLFA, with highest values in intensive-permanent grasslands (Bittman et al., 2005; Deru et al., 2023). In contrast, we found

Table 2
Abiotic soil parameters sorted for intensive-permanent, multi-species, and semi-natural grasslands (n = 12 for each management type). ANOVA *p*-values are given, with significant results ($p < 0.05$) in bold. Parameter means with different superscript letters indicate significant differences according to post-hoc Tukey tests ($p < 0.05$).

		Grassland management type						
		Intensive-permanent		Multi-species		Semi-natural		
Parameter	Unit	Mean	s.d.	Mean	s.d.	Mean	s.d.	*p*-value
pH_{KCl} (0–10 cm)	–	5.78^{b}	0.51	5.67^{b}	0.45	4.80^{a}	0.36	**<0.001**
P_{AL}	mg P kg $soil^{-1}$	39.8	18.4	38.9	20.2	32.3	17.3	0.480
Plant available K	mg K kg $soil^{-1}$	92.5^{b}	55.5	88.4^{b}	79.2	36.5^{a}	19.9	**0.001**
SOM (0–10 cm)	%	5.49	1.26	4.77	1.27	5.36	1.00	0.135
SOM (0–30 cm)	%	4.37	1.00	4.60	1.20	4.08	1.17	0.337
C_{total} (0–10 cm)	%	2.83	0.83	2.47	0.75	2.68	0.64	0.269
C_{total} (0–30 cm)	%	2.15	0.54	2.24	0.66	1.99	0.65	0.481
N_{total} (0–10 cm)	mg N kg $soil^{-1}$	2737^{b}	597	2108^{a}	533	2357^{ab}	504	**0.037**
Potentially mineralisable N	mg N kg $soil^{-1}$	82.4^{b}	11.8	65.8^{a}	13.1	83.1^{ab}	23.3	**0.009**
C:N ratio	–	10.33	1.98	11.60	1.32	11.48	2.16	0.130
Field water content (5–10 cm)	Vol%	23.7^{ab}	3.4	21.6^{a}	3.5	25.5^{b}	5.0	**0.043**
Bulk density (5–10 cm)	g cm^{-3}	1.45^{ab}	0.06	1.50^{b}	0.08	1.40^{a}	0.05	**0.002**

Table 3
Microbial parameters and protozoa for intensive-permanent, multi-species, and semi-natural grasslands (n = 12 for each management type). ANOVA *p*-values are given, with significant results ($p < 0.05$) in bold. Parameter means with different letters indicate significant differences according to post-hoc Tukey tests ($p < 0.05$).

		Grassland management type						
		Intensive-permanent		Multi-species		Semi-natural		
Parameter	Unit	Mean	s.d.	Mean	s.d.	Mean	s.d.	*p*-value
Bacteria	µg PLFA g $soil^{-1}$	17.83^{ab}	4.21	13.42^{a}	3.90	21.75^{b}	7.15	**0.003**
Actinomycetes	µg PLFA g $soil^{-1}$	2.02^{ab}	0.57	1.50^{a}	0.47	2.31^{b}	0.80	**0.012**
Saprotrophic fungi	µg PLFA g $soil^{-1}$	0.70	0.20	0.60	0.22	0.83	0.31	0.084
Fungal:bacterial ratio	–	0.04	0.01	0.05	0.03	0.04	0.02	0.647
Protozoa	µg PLFA g $soil^{-1}$	0.31^{b}	0.09	0.23^{a}	0.07	0.30^{ab}	0.05	**0.033**

Table 4

Nematode community parameters for intensive-permanent, multi-species, and semi-natural grasslands (n = 12 for each management type). ANOVA *p*-values are given, with significant results ($p < 0.05$) in bold. Parameter means with different letters indicate significant differences according to post-hoc Tukey tests ($p < 0.05$).

		Grassland management type						
		Intensive-permanent		Multi-species		Semi-natural		
Parameter	Unit	Mean	s.d.	Mean	s.d.	Mean	s.d.	*p*-value
Nematode abundance	N 100 g soil^{-1}	3785	1582	3404	1374	3159	1348	0.570
Nematode γ diversity (genus)	N	46		45		46		
Nematode α diversity (genus)	N	19.8	3.2	21.6	4.5	21.4	4.1	0.339
Nematode ENS[*] (N1)	N	6.6	2.2	7.9	2.3	8.6	2.7	0.104
Channel Index		9.4	7.6	10.0	10.6	10.1	9.8	0.979
Enrichment Index		70.5	19.6	67.3	20.3	71.7	18.7	0.850
Structure Index		68.5	12.3	66.0	9.0	73.9	9.4	0.172
Herbivores	%	35.5	10.8	37.3	15.8	29.1	14.7	0.305
Ectoparasites	% of herb.	61.2	18.0	57.6	23.7	45.1	23.8	0.070
Epidermal feeders	% of herb.	26.6^{a}	12.3	21.2^{a}	17.7	39.9^{b}	25.1	**0.009**
Fungivores	%	5.9	4.0	4.6	3.0	7.1	5.2	0.252
Bacterivores	%	47.8	13.0	46.1	18.7	49.7	15.1	0.856
Omnivores	%	10.2	3.1	11.0	6.3	12.3	4.7	0.553
Predators	%	0.7	1.1	1.0	1.3	1.9	1.5	0.062
Herbivore Footprint	μg C ind^{-1} LT^{-1} [**]	734^{b}	590	696^{b}	576	193^{a}	187	**0.020**
Fungivore Footprint	μg C ind^{-1} LT^{-1}	21^{b}	11	13^{a}	7	32^{b}	32	**0.035**
Bacterivore Footprint	μg C ind^{-1} LT^{-1}	1225	1052	1295	1783	1077	938	0.918
Omnivore Footprint	μg C ind^{-1} LT^{-1}	380	176	330	147	395	140	0.569
Predator Footprint	μg C ind^{-1} LT^{-1}	26	49	30	34	54	37	0.175

[*] Effective Number of Species based on Hills number N1, i.e. the exponent of the Shannon.

[**] Life Time amount of metabolised carbon per individual, expressed in micrograms of carbon.

Table 5

Micro-arthropod parameters for intensive-permanent, multi-species and semi-natural grasslands (n = 12 for each management type). ANOVA *p*-values are given, with significant results ($p < 0.05$) in bold. Parameter means with different letters indicate significant differences according to post-hoc Tukey tests ($p < 0.05$).

		Grassland management type						
		Intensive- permanent		Multi-species		Semi-natural		
Parameter	Unit	Mean	s.d.	Mean	s.d.	Mean	s.d.	*p*-value
Microarthropod abundance	N m^{-2}	26,895	24,244	37,840	32,695	28,388	20,793	0.500
Microarthropod γ diversity (family)	N	30		27		39		
Microarthropod α diversity (family)	N	11.8^{a}	2.6	11.3^{a}	2.2	15.3^{b}	4.6	**0.012**
Microarthropod ENS[*] (N1; family)	N	5.4^{a}	1.9	5.2^{a}	2.0	8.5^{b}	3.5	**0.002**
Fungivorous browsers	N m^{-2}	16,811	19,485	26,677	27,075	13,970	12,441	0.269
Fungivorous grazers	N m^{-2}	279^{a}	605	0^{a}	0	2348^{b}	2623	**0.008**
Herbofungivores	N m^{-2}	1740	2267	1409	2204	2484	2783	0.346
Herbivorous browsers	N m^{-2}	520^{a}	568	1271^{ab}	1464	1895^{b}	1794	**0.018**
Herbivorous grazers	N m^{-2}	3522	2385	3196	1841	3439	1791	0.951
Omnivores	N m^{-2}	2148	1438	3029	2283	2168	1401	0.367
Predators	N m^{-2}	1876	2207	2200	1498	2084	1758	0.896

[*] Effective Number of Species based on Hills number N1, i.e. the exponent of the Shannon.

Table 6

Earthworm parameters for intensive-permanent, multi-species and semi-natural grasslands (n = 12 for each management type). ANOVA *p*-values are given, with significant results ($p < 0.05$) in bold. Parameter means with different letters indicate significant differences according to post-hoc Tukey tests ($p < 0.05$).

		Grassland management type						
		Intensive-permanent		Multi-species		Semi-natural		
Parameter	Unit	Mean	s.d.	Mean	s.d.	Mean	s.d.	*p*-value
Earthworm abundance	n m^{-2}	583	310	515	246	562	442	0.886
Earthworm biomass	G	189	104	157	82	130	68	0.285
Earthworm MIB[*]	G	0.32	0.10	0.33	0.17	0.28	0.10	0.450
Earthworm γ diversity (species)	N	7		8		9		
Earthworm α diversity (species)	N	4.3	1.4	3.4	1.0	3.7	1.5	0.266
Earthworm ENS[**] (N1; species)	N	2.6	0.6	2.3	0.7	2.6	0.7	0.334
Epigeic earthworms	n m^{-2}	88^{ab}	76	47^{a}	50	121^{b}	6	**0.032**
Endogeic earthworms	n m^{-2}	423	264	411	218	295	371	0.974
Anecic earthworms[***]	n m^{-2}	28	45	19	34	1	4	0.113

[*] Mean Individual Biomass.

[**] Effective Number of Species based on Hills number N1, i.e. the exponent of the Shannon.

[***] With the method used, soil cubes of up to 20 cm depth, anecic species such as *Lumbricus terrestris* are underrepresented in the sample.

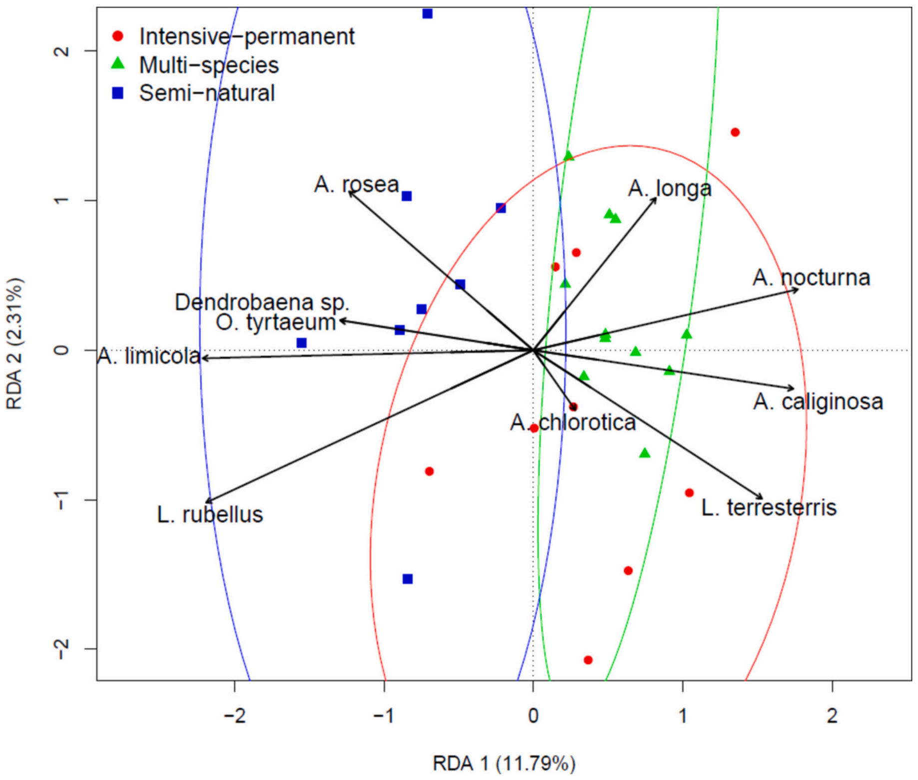

Fig. 3. RDA biplot showing the first and second redundancy axes of the earthworm species abundance residuals. Only species vectors that explained >5 % variation were included. Variation between blocks (32.52 %) has been conditioned for in the analysis. Grassland management types (intensive-permanent, multi-species, and semi-natural grasslands) are indicated by different colours and ellipses with 95 % confidence levels. Monte Carlo permutation testing showed that the model was significant ($p = 0.010$), and explained 57.96 % of total variation after conditioning. The first RDA axis was significant ($p = 0.002$) whereas the second axis was not considered significant in explaining total variation ($p = 0.854$).

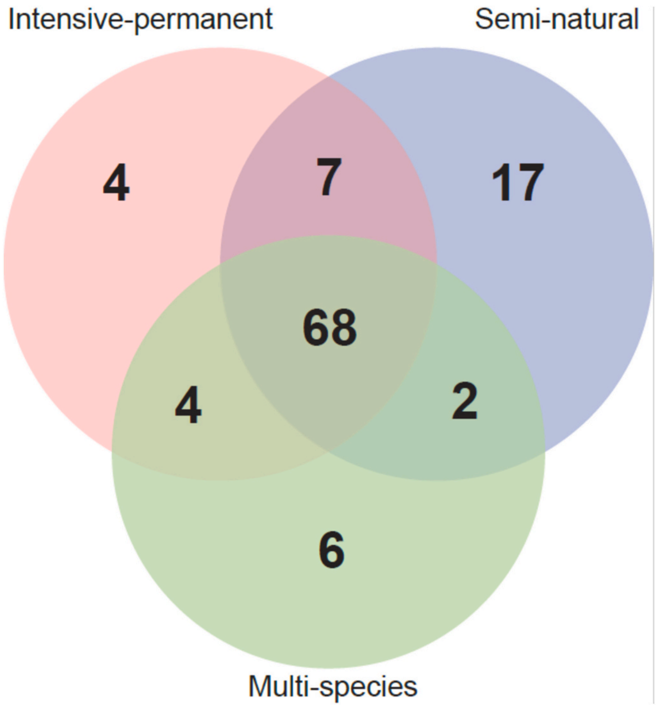

Fig. 4. Gamma diversity of soil biota (nematodes, micro-arthropods and earthworms) for the three grassland management types.

significantly higher amounts of total bacterial and actinomycete PLFA in the semi-natural grasslands than in the multi-species grasslands; intensive-permanent grasslands having an intermediate position. Similarly, Van Eekeren et al. (2009a, 2009b) and De Vries et al. (2006) did not find an effect of fertilisation on bacterial biomass in sandy soil. The lower bacterial biomass in multi-species grasslands than in intensive-permanent and semi-natural grasslands in our experiment may well be partly explained by the lower number of years since last tillage and the negative effect of tillage on SOM (non-significant), N-total and potentially-mineralisable N (both significant) in the 0–10 cm layer (Van Eekeren et al., 2008; Hassink et al., 1991). Both total bacterial and actinomycete PLFA were correlated with potentially mineralisable N, N-total and number of years since last tillage. The higher soil nitrogen status in the 0–10 cm soil layer in intensive-permanent grasslands is most probably determined by the number of years since last tillage and fertilisation (Van Eekeren et al., 2008; Van Eekeren et al., 2009b) whereas this higher soil nitrogen status in semi-natural grasslands is likely to be caused by the number of years since last tillage.

As hypothesised, the amount of saprotrophic fungal PLFA tended to be higher in semi-natural grasslands ($p = 0.084$) than in multi-species grassland, which agrees with observations that fungi are better adapted to environments with more stabilised and recalcitrant organic matter (Paterson et al., 2008). However, the absence of a difference in fungi between intensive-permanent grasslands and semi-natural grasslands was unexpected and in contrast with previous results which showed higher fungal biomass in less fertilised and more extensively managed grasslands (De Vries et al., 2006, 2007, 2012). Similar to bacteria, disturbance due to tillage may also have played a role, besides differences in organic matter quantity and quality. Tillage occurred well before the sampling of the intensive-permanent grasslands and the semi-natural grasslands, averaging 8.3 and 21.3 years, respectively (Table 1).

Although we did not find a direct correlation between the numbers of years since last tillage and saprothrophic fungi in our experiment, the influence of tillage could be both direct as it damages the mycelial network (Beare et al., 1997; Frey et al., 1999) and indirect via the influence on SOM and soil nitrogen status.

Protozoan PLFA was highest on intensive-permanent grasslands and correlated to saprothrophic fungal PLFA, total bacterial PLFA and actinomycete PLFA. Most protozoa are heterotrophic and known to graze on bacteria, yeast, fungi and algae.

In conclusion, for the soil microbes the differences between the three grassland management types seem to be more affected by the disturbance due to tillage during the establishment phase than by fertilisation and plant species composition. The influence of this disturbance could be direct, due to the damage of tillage on the microbes, and indirect, via the influence on SOM-related parameters such as N_{total} and potentially mineralisable N. The amount of protozoan PLFA followed the microbial PLFA.

4.2. Nematodes

With an average of 3159–3785 nematodes per 100 g^{-1} soil for the three grassland management types, the abundance of nematodes was lower than the national reference for semi-natural grasslands on sandy soils (5024 nematodes per 100 g^{-1}, Rutgers et al., 2009). Other research on sandy soil measured a three-year average of 6463 and 6222 nematodes per 100 g^{-1} on intensive-permanent and temporary grasslands, respectively (Van Eekeren et al., 2008). There are no plausible explanations for the difference in abundance, since Van Eekeren et al. (2008) also sampled in the autumn. However, the number of genera in the three grassland management types (range from 19.4 to 21.8) were comparable to the number on intensive-permanent and temporary grasslands (range 19.4–21.6) measured by Van Eekeren et al. (2008).

As hypothesised the carbon footprint of herbivores was higher in intensive-permanent and multi-species grasslands than in semi-natural grasslands, because of the higher aboveground biomass produced in these two grassland management types (Verschoor, 2002).

As hypothesised the carbon footprint of fungivorous nematodes was higher in semi-natural and intensive-permanent grasslands than in multi-species grasslands. This could be explained by the correlation of fungivorous nematodes with the amount of PLFA for saprotrophic fungi. The higher percentage of epidermal and root-hair feeders in the semi-natural grasslands could even strengthen this hypothesis, because part of the epidermal and root-hair feeders are also classified as facultative fungivorous (Du Preez et al., 2022).

We hypothesised a difference in the nematode enrichment and structure index, but did not find differences between the three grassland management types. In the functional guild classification of Ferris et al. (2001), all three grassland management types were classified in quadrant B, which is characterised as matured, N-enriched, low C-to-N ratio, and bacterial-dominated. With the more fungal-dominated food web in the semi-natural grasslands and their lower primary productivity (Zetterlind et al., submitted), we expected to find lower enrichment index values in the semi-natural grasslands, which would then result in a shift in the nematode community towards quadrant C, i.e. matured, fertile, moderate C-to-N ratio, and more bacterial- and fungal-balanced. Our data suggest that there were no differences in functional guild composition between the grassland management systems. However, at the genus level we did find significant differences in nematode community structure (Fig. 1) whereby the first axis of the RDA of nematode genera was positively correlated with aboveground biomass production and negatively with number of plant species, number of years since last tillage, and saprotrophic fungal PLFA.

In conclusion, the abundance of nematodes was not different for the three grassland types. However, the taxonomic diversity of nematodes translated into a nematode community with a functional diversity characterised by a positive relationship between the aboveground biomass production and herbivorous nematodes and fungal biomass and fungivorous nematodes.

4.3. Micro-arthropods

With an average of 26,895–37,840 micro-arthropods per m^{-2} for the three grassland management types, the abundance of micro-arthropods was lower than the national reference for permanent grasslands (46,000 micro-arthropods per m^{-2}) and semi-natural grasslands on sandy soils (101,000 micro-arthropods per m^{-2}) (Rutgers et al., 2009). Recent research in an adjacent area on intensive-permanent and semi-natural grasslands on sandy soils, sampled in autumn 2019, also reported higher but not significant differences in abundance between intensive-permanent grasslands and semi-natural grasslands (70,500 and 94,000 micro-arthropods per m^{-2}, respectively) (Van Eekeren et al., 2022). Soil disturbance by tillage and trampling by grazing is one of the main causes of decline in micro-arthropod populations, and this is explained by a redistribution of organic matter and changes in the temperature, humidity and pore-size distribution in the soil microhabitat (Loring et al., 1981; Blevins et al., 1984; Perdue and Crossley, 1990; Van Eekeren et al., 2022). For example, grazing can cause compaction in the upper soil layer, which makes the micro-arthropods more susceptible to desiccation in sandy soils. We found no relationship between number of years since last tillage and the abundance of micro-arthropods. Van Eekeren et al. (2022) found this relationship only for agricultural and semi-natural grasslands with mowing management, whereby a mown agricultural grassland which had been tilled 39 years before sampling had the highest abundance. Siepel (1996) reported >200,000 micro-arthropods per m^{-2} in semi-natural grasslands which for decades had not been tilled and only mown. The mixed management of mowing and grazing in our intensive-permanent and multi-species grasslands as well as the relatively young grasslands may explain the lower numbers of micro-arthropods found in our study.

Although low in numbers, we found a higher abundance of fungivorous grazers in semi-natural grasslands. Micro-arthropods play a role in the decomposition of organic matter and in nutrient recycling. More specifically, Siepel and Maaskamp (1994) suggest that both fungivorous and herbofungivorous grazers have a stimulating effect on microbial respiration, while fungivorous browsers have an inhibiting effect. The fungivorous browsers in our experiment were numerically lower for semi-natural grasslands in our experiment. We did not find a relationship between either fungivorous grazers or browsers and saprotrophic fungal PLFA, like we found for fungivorous nematodes. More fungivorous grazers were also found by Van Eekeren et al. (2022) in semi-natural grasslands with pure mowing management. This higher abundance of fungivorous grazers may also be explained by a combination of a higher number of years since last tillage and the management of only mowing on semi-natural grasslands. We found a significant correlation between the abundance of fungivorous grazers and the number of years since last tillage. Although the bulk density of the soil was lower for semi-natural grasslands, we found no relationship between the abundance of fungivorous grazers and bulk density or grazing days.

In line with our hypotheses, we found the highest diversity of micro-arthropods in the semi-natural grasslands. This is in agreement with Van Eekeren et al. (2022), who compared intensive-permanent and semi-natural grasslands. Moreover, it is supported by the correlations between site scores on the first RDA axis (describing the variation in micro-arthropod family abundances) and different parameters which determine the differences between semi-natural grasslands on the one hand and intensive-permanent and multi-species grasslands on the other.

In conclusion, the abundance of micro-arthropods was not different for the three grassland types. However, the taxonomic diversity of micro-arthropods was higher in the semi-natural grasslands, and both the taxonomic and functional diversity in semi-natural grasslands differed significantly from intensive-permanent and multi-species grasslands.

4.4. Earthworms

With an average of 515–583 earthworms per m^{-2} in the three grassland types, the abundance of earthworms was much higher than the national reference for permanent grasslands (187 earthworms per m^{-2}) and semi-natural grasslands on sandy soils (133 earthworms per m^{-2}) (Rutgers et al., 2009). The range of average earthworm biomass of 130–189 g per m^{-2} was comparable to the three-year average of 166 g per m^{-2} in intensive-permanent grasslands on a sandy soil (Van Eekeren et al., 2008). The difference in abundance can be due to the time of sampling, which was in spring for the national reference and in autumn for this research as well as for the experiment in Van Eekeren et al. (2008).

In contrast to our hypothesis of a predominance of earthworms in the intensive-permanent and multi-species grasslands, we found no differences in earthworm abundance. Earthworm abundance and biomass in grassland are found to be influenced by soil disturbance, as reflected in number of years since last tillage (Van Eekeren et al., 2008; Postma-Blaauw et al., 2012), fertilisation level and type (Van Eekeren et al., 2009b; Edwards and Arancon, 2022; Deru et al., 2023; Torppa et al., 2024), botanical composition and the presence of legumes (Van Eekeren et al., 2009a), and soil properties such as water content, N_{total}, C-to-N ratio and pH (De Wandeler et al., 2016; Deru et al., 2018; Deru et al., 2023; Torppa et al., 2024). The similarities in earthworm abundance may be explained by interactions of several factors inherent to the treatments of the three grassland management types. An example is pH and soil water content. We expected the lower pH in semi-natural grasslands to reduce the abundance of earthworms, but we did not find a correlation between pH in the 36 grasslands and the different earthworm parameters. On the other hand, we expected the higher water content in semi-natural grasslands to increase the abundance of earthworms; however, we did not find a correlation between the water content in the 36 grasslands and the different earthworm parameters. For the semi-natural grasslands, the years under extensive management may also have been of importance. On twelve sites on clay soils, Jansma et al. (2024) measured lower earthworm biomass in semi-natural grasslands than in intensive-permanent grasslands, but only if they had been under nature management for >25 years, and not if intensive management had stopped only 5–15 years ago. In our experiment, two of the twelve semi-natural grasslands had been under extensive management for >25 years, but only one of these had a lower earthworm biomass (53 and 234 g per m^{-2} versus an average of 128 g per m^{-2} for the other 10 semi-natural grasslands).

The number of 4.3 taxa in the intensive-permanent grasslands was comparable to the Dutch reference of 4.6, but the taxa richness of 3.7 in the semi-natural grasslands was lower than the national reference of 6.8 for sandy soils (Rutgers et al., 2009). The significantly higher abundance of epigeic earthworms in the semi-natural grasslands may be related both to the fact that the semi-natural grasslands are mown later in the growing season, thus leaving more residue in the field, which is the preferred food for epigeic earthworms (Doube et al., 1997), and to the absence of trampling, to which these species are susceptible (Cluzeau et al., 1992). Bulk density was lowest in the semi-natural grasslands in our experiment.

Although a lower abundance of earthworms for semi-natural grasslands was hypothesised, there was no difference between the earthworm parameters *total abundance*, *biomass* and *number of taxa* of the three grassland management types. This may be related to an interaction of factors (e.g. soil parameters and management) inherent to the treatments. For example, semi-natural grasslands had lower pH (negative for earthworms) but higher soil water content (positive for earthworms), which may have compensated each other. Semi-natural grasslands had a higher abundance of epigeic earthworms, and this might be related to the management, which was mainly mowing with a low frequency, resulting in more aboveground plant residue as food source and less trampling by animals resulting in a lower bulk density.

5. Conclusions

Between the three grassland management types, i.e. intensive-permanent, multi-species, and semi-natural grasslands, no differences were found in the total abundance and biomass of nematodes, micro-arthropods and earthworms when measured at the end of the growing season, partially refuting our first hypothesis. Bacterial and actinomycete PLFA were higher in semi-natural grasslands than in multi-species grasslands; permanent grasslands took an intermediate position. While the higher actinomycete PLFA was expected, the bacterial PLFA did not align with our hypothesis. The number of years since last tillage and/or the permanent character of the semi-natural and intensive-permanent grasslands showed a positive relationship with these microbial parameters. Tillage may influence microbial parameters directly due to damage on fungal hyphae and indirectly via the influence on the food availability of microbes via the negative effect of tillage on SOM and related parameters.

The grassland management type had an effect on alpha diversity only in the case of micro-arthropods with higher taxonomic richness in semi-natural grasslands. This partially refutes our second hypothesis, which anticipated significant differences across other soil biota. In addition, gamma diversity of all taxonomic groups taken together was higher for the semi-natural grasslands, again mainly because of a higher micro-arthropod taxonomic richness, therefore accepting our third hypothesis. Nematode, micro-arthropod and earthworm taxonomic community structure differed between grassland management types; this was most distinct in the case of nematodes and micro-arthropods, with the largest differences between semi-natural and multi-species grasslands. In general, we observed a trend of a more bacterial-based food web with protozoa in the intensive-permanent grasslands, and a more fungal-based food web with micro-arthropods in the semi-natural grasslands, consistent with our fourth hypothesis. The soil food web of the multi-species grasslands differed most from the other two grassland types and was more herbivory-based than the semi-natural grasslands. The differences in abundance and diversity of soil biota between intensive-permanent and multi-species grasslands appeared to be most affected by the disturbance from tillage and its direct and indirect effects on soil biota and its living conditions.

We conducted this research on sandy soils and not on clay or peat soils because it is the major soil type in The Netherlands. On this soil type, we expected the greatest differences between intensive-permanent grasslands and semi-natural grasslands because of a gradient in soil fertility parameters (e.g. pH, SOM and soil nutrients), land use intensity and vegetation. Apparently, these differences were not great enough and/or permanent land use without tillage in both intensive-permanent and semi-natural grasslands turns out to be a dominant factor. Jansma et al. (2024) and Deru et al. (2018) show in clay and peat soils, respectively, that for semi-natural grasslands the years under extensive management (> 25 years) may play a role. The factor of disturbance through tillage, especially with multi-species grasslands, will also have an important effect on the soil biodiversity in clay and peat soils.

For farmers who wish to make an integral decision on the transition of grasslands into future farming systems, it is important to also take into account the effect of this transition on the aboveground biodiversity of insects and birds, along with other ecosystem services such as production (Zetterlind et al., submitted). Both multi-species and semi-natural grasslands may be an option, in particular for grassland systems that simultaneously utilise and support soil biodiversity and their ecosystem services. However, in the case of multi-species grasslands, longevity without tillage is an important point of attention.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.apsoil.2025.105881.

CRediT authorship contribution statement

Nick van Eekeren: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. **Jurre Dekker:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Rob Geerts:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation, Conceptualization. **Pedro Janssen:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Anthonie Stip:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Tim Visser:** Writing – review & editing, Writing – original draft, Methodology, Funding acquisition, Conceptualization. **Jaap Bloem:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis, Data curation. **Ron de Goede:** Writing – review & editing, Writing – original draft, Supervision, Methodology, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Riekje Bruinenberg, Joachim Deru, Wim Dimmers, Harm Keidel, Roos van de Logt and Esther Wattel for their assistance with the soil sampling and species identification. In addition, we would like to thank the 24 farmers and land-owners who allowed us to use their fields for this research. This study was financed by DairyNL (ZuivelNL; organisation of the Dutch dairy supply chain), Rabobank, Triodos Foundation, World Wide Fund for Nature and the Dutch Ministry of Agriculture, Nature, and Food Quality as part of the Public Private Partnership 'Sustainable Dairy Chain' (LWV19256; project living lab C2: multi-species and semi-natural grasslands).

Data availability

Data will be made available on request.

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